

## **REMARKS**

The present application relates to hybrid maize plant and seed 39R34. Claims 9-11, 13-19, 22-24, 26-32, 34-40 have been canceled. Claims 43-60 have been added. No new matter has been added by the present amendment. Applicant respectfully requests consideration of the following remarks.

### **Detailed Action**

#### *A. Status of the Application*

Applicant acknowledges the objections to claims 1, 5-7, 12, 16, 25 and 29 as withdrawn in light of the claim amendments and Applicant's assurances that the ATCC accession number for seed of 39R34 will be inserted at time of allowance. Applicant further acknowledges the rejections of claims 1-10, 12-14, 16-18, 20-23, 25-27 and 29-31 under 35 U.S.C. § 112, second paragraph, as withdrawn. The rejection of claims 1-32 under 35 U.S.C. § 112, first paragraph, requiring deposit of seed of plant 39R34, are acknowledged as withdrawn.

#### *B. Specification*

Applicant submits the Deposit section on page 40 has been amended in order to properly include both the hybrid maize plant 39R34 and the inbred parents GE533276 and GE533139 within the Deposit paragraph. The changes do not add new matter as there is literal support for the minor changes on pages 7 in the originally filed specification. The specification has now been amended to correct these minor changes.

In addition, Applicant respectfully submits that the actual ATCC deposit of the two inbred plants will be delayed until the receipt of notice that the application is otherwise in condition for allowance, in compliance under 37 C.F.R. §§ 1.801-1.809. Once such notice is received, an ATCC deposit will be made, and the specification will be amended to contain the accession number of the deposit, the date of the deposit, a description of the deposited biological material sufficient to specifically identify it and to permit examination and the name and address of the depository. The claims will also be amended to recite the ATCC deposit number. Applicant submits that at least 2,500 seeds of hybrid maize plant 39R34 and the inbred parents GE533276 and GE533139 will be deposited with the ATCC. Applicant further asserts that the deposits will be made without restriction.

*C. Claim Objections*

The Examiner objects to claim 42 under 37 C.F.R. § 1.75(c) as being of improper dependent form. The Examiner states that claim 42 attempts to limit the method of claim 41 by requiring a male sterile maize plant to be produced, but the step of producing the plant is inherent to the method of claim 41. Applicant has now amended claim 42 to be rewritten as follows: --A male sterile maize plant produced by the method of claim 41.--, thus alleviating this rejection.

**Rejections Under 35 U.S.C. § 112, Second Paragraph**

Claims 11, 15, 19, 24, 28 and 32 remain rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention, for the reasons of record stated in the Office Action mailed August 29, 2002. The Examiner states the specification does not define when one would use the particular adjective to recite the degree of expression of a trait in Tables 1-4 and when one would not. The Examiner further states that even if "excellent" is the most precise definition that can be used, it remains unclear when a trait should be defined with that adjective versus "good".

Applicant has canceled claims 11, 15, 19, 24, 28 and 32, thereby alleviating this rejection.

Claims 8, 11, 15, 19, 21, 24, 28, 32 and 38-42 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Claims 8, 21 and 41 stand rejected for the recitation "genetic factor" as rendering the claims indefinite.

Applicant has now amended claims 8 and 21 to delete the terminology "genetic factor" and include the recitation --introgressing a cytoplasmic gene--, and amended 41 to delete the terminology "genetic factor" and include the recitation --transgene--, as suggested by the Examiner, thus alleviating this rejection. Applicant thanks the Examiner for this suggested language.

The Examiner rejects claims 11, 15, 19, 24, 28, 32, 38 and 39 for the recitations "has derived at least 50% of its alleles" in claims 11, 15, 19, 24, 28 and 32, and "deriving at least 50% of its alleles" in claims 38 and 49 rendering the claims indefinite.

Applicant has canceled claims 11, 15, 19, 24, 28, 32, 38 and 39, alleviating this rejection.

Claim 38 stands rejected as indefinite for the recitation "on average, deriving at least 50%" in line 2.

Applicant has canceled claim 38, thereby alleviating this rejection.

The Examiner rejects claim 39 as indefinite for the recitation "A 39R34 maize plant selected from the population of 39R34 progeny maize plants".

Applicant has canceled claim 39, thus alleviating this rejection.

Claim 40 stands rejected for the recitation "further comprising applying double haploid methods" as rendering the claim indefinite. The Examiner states the recitation broadens the scope of parent claim 37, which only involves crosses and does not encompass any double haploid method.

Applicant has canceled claim 40, alleviating this rejection.

In light of the above amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

#### **Rejections Under 35 U.S.C. § 112, First Paragraph**

Claims 9-19 and 22-32 remain and claims 34-40 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention, for the reasons of record stated in the Office Action mailed August 29, 2002.

Applicant respectfully traverses this rejection. Applicant asserts that the introgression of mutant genes and transgenes is easily, routinely and extensively practiced by those of ordinary skill in the art. Backcrossing has been known since the 1920's and, because of its predictability, is the method preferred by commercial plant breeders to introduce transgenes into already developed and tested material. As stated in Poehlman *et al.* (1995) on page 334, submitted in the Information Disclosure Statement, a backcross derived inbred line fits into the same hybrid combination as the recurrent parent inbred line and contributes the effect of the additional gene added through the backcross." Applicants further point out that cytoplasmic male sterility genes do not introduce linked nuclear genes. Wych (1988) on page 585-586, submitted in the Information Disclosure Statement, discusses how the male sterility trait is routinely backcrossed into an inbred line and how this is used to produce a sterile/fertile blend of an F1 hybrid in order

to reduce seed production costs. In fact, many commercial products are produced in this manner, and those of ordinary skill in the art consider the F1 hybrid produced with the male sterile inbred to be the same variety as the F1 hybrid produced with the fertile version of the inbred.

Applicants also refer the Examiner to Openshaw *et al.* submitted herewith, which states the "the backcross breeding procedure is being used widely to transfer simply inherited traits into elite genotypes...Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm."

Nonetheless, in an effort to expedite prosecution, Applicant has canceled claims 9-11, 13-19, 22-24, 26-32, 34-40 and added new claims 43-60, thereby alleviating the rejection. In addition, Applicant has now amended claims 12 and 25 to include --contains one or more mutant genes or transgenes which have been introgressed therein, said mutant genes or transgenes selected from the group consisting of: a plant disease resistance gene, an insect resistance gene, a herbicide resistance gene, and a male sterility gene--, thereby limiting the claims to the types of transgenes that may be introduced and that are supported by the specification on pages 32-38, as suggested by the Examiner. Applicant therefore respectfully requests withdrawal of the above rejections.

Claim 33 stands rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is more nearly connected, to make and/or use the invention. The Examiner states the claim is drawn towards a method of making a hybrid plant designated 39R34 comprising crossing inbred maize plants GE533276 and GE533139, however the Examiner states the terms of this deposit are not known.

Applicant respectfully traverses this rejection. Applicant herein submits the Deposits section has been amended in order to properly include both the hybrid maize plant 39R34 and the inbred parents GE533276 and GE533139 within the Deposit paragraph on page 40. The changes do not add new matter as there is literal support for the minor changes on page 7 in the originally filed specification. The specification has now been amended to correct these minor changes. Applicant thanks the Examiner for pointing out this inadvertent mistake.

In addition Applicant submits that the actual ATCC deposit will be delayed until receipt of notice that the application is otherwise in condition for allowance. As provided in 37 C.F.R. §§ 1.801-1.809, Applicant wishes to reiterate they will refrain from deposit of hybrid 39R34 and

inbred parents GE533276 and GE533139 until allowable subject matter is indicated. Once such notice is received, an ATCC deposit will be made, and the specification will be amended to contain the accession number of the deposit, the date of the deposit, description of the deposited biological materials sufficient to specifically identify and to permit examination and the name and address of the depository. The claims will also be amended to recite the proper ATCC deposit numbers. The Applicant provides assurance that:

- a) during the pendency of this application access to the invention will be afforded to the Commissioner upon request;
- b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;
- c) the deposit will be maintained in a public depository for a period of thirty years, or five years after the last request for the enforceable life of the patent, whichever is longer;
- d) a test of the viability of the biological material at the time of deposit will be conducted (see 37 C.F.R. § 1.807); and
- e) the deposit will be replaced if it should ever become inviable.

Therefore, Applicant submits at least 2500 seeds of hybrid maize plant 39R34 and the inbred parents GE533276 and GE533139 will be deposited with the ATCC. In view of this assurance, the rejection under 35 U.S.C. § 112, first paragraph, should be removed. (MPEP § 2411.02) Such action is respectfully requested.

In light of the above amendments and remarks, Applicant respectfully requests reconsideration and withdrawal of the rejections to claims 9-19 and 22-40 under 35 U.S.C. § 112, first paragraph.

#### **Issues Under 35 U.S.C. § 102/103**

Claims 11, 15, 19, 24, 28 and 32 remain and new claims 36, 38 and 39 stand rejected under 35 U.S.C. § 102(e) as anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as obvious over Kevern (U.S. Patent No. 6,242,673), for the reasons of record stated in the Office Action mailed August 29, 2001. The Examiner acknowledges that Applicant's arguments were found persuasive for the withdrawal of the rejection from claims 1-10, 12-14, 16-18, 20-23, 25-27 and 29-32.

Applicant respectfully traverses this rejection. However, in order to expedite prosecution, Applicant has now canceled claims 11, 15, 19, 24, 28, 32, 36, 38, and 39, thus alleviating this rejection. Applicant respectfully requests the Examiner withdraw the rejections to claims 11, 15, 19, 24, 28, 32, 36, 38 and 39 under 35 U.S.C. § 102(e) as anticipated by or, in the alternative, under 35 U.S.C. §103(a) as obvious over Kevern (U.S. Patent No. 6,242,673).

### **Conclusion**

In conclusion, Applicant submits in light of the above amendments and remarks, the claims as amended are in a condition for allowance, and reconsideration is respectfully requested. If it is felt that it would aid in prosecution, the Examiner is invited to contact the undersigned at the number indicated to discuss any outstanding issues.

This is a request under the provision of 37 C.F.R. § 1.136(a) to extend the period for filing a response in the above identified application for two months from May 10, 2003 to July 10, 2003. Applicant is a large entity; therefore, a check is enclosed in the amount of \$410.00 for two months to cover the cost of the extension.

Any deficiency or overpayment should be charged or credited to Deposit Account 26-0084.

Reconsideration and allowance is respectfully requested.

Respectfully submitted,



LILA A.T. AKRAD, Reg. No. 52,550  
McKEE, VOORHEES & SEASE  
801 Grand Avenue, Suite 3200  
Des Moines, Iowa 50309-2721  
Phone No. (515) 288-3667  
Fax No. (515) 288-1338  
**CUSTOMER NO: 27142**

Attorneys of Record

- pw/LA -

# Marker-assisted Selection in Backcross Breeding

S.J. Openshaw

Pioneer Hi-Bred Intl. Inc., P.O. Box 1004, Johnston, IA 50131

S.G. Jarboe<sup>1</sup>

CIMMYT, Lisboa 27, Apdo. Postal 6-641, 06600 Mexico, D.F., Mexico

W.D. Beavis

Pioneer Hi-Bred Intl. Inc., P.O. Box 1004, Johnston, IA 50131

**Abstract.** The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Genetic markers can increase the effectiveness of backcrossing by 1) increasing the probability of obtaining a suitable conversion, and 2) decreasing the time required to achieve an acceptable recovery. Simulation and field results indicated that, for a genome consisting of ten 200-cM chromosomes, basing selection on 40 or 80 markers in 50 BC individuals that carry the allele being transferred can reduce the number of backcross generations needed from about seven to three.

The backcross breeding procedure has been used widely to transfer simply inherited traits into elite genotypes. Usually, the trait being transferred is controlled by a single gene, but highly heritable traits that are more complexly inherited have also been transferred successfully by backcrossing; for example, maturity in maize (Rinke and Sentz, 1961; Shaver, 1976). Today, backcrossing is being used to transfer genes introduced by such techniques as transformation or mutation into appropriate germplasm.

Several plant breeding textbooks give good descriptions of the backcross procedure (Allard, 1960; Fehr, 1987). A donor parent (DP) carrying a trait of interest is crossed to the recurrent parent (RP), an elite line that is lacking the trait. The  $F_1$  is crossed back to the RP to produce the  $BC_1$  generation. In the  $BC_1$  and subsequent backcross generations, selected individuals carrying the gene being transferred are backcrossed to the RP. The expected proportion of DP genome is reduced by half with each generation of backcrossing. Ignoring effects of linkage to the selected DP allele being transferred, the percentage recurrent parent (%RP) genome expected in each backcross generation is calculated as:

$$\%RP = 100 [1 - (0.5)^{n+1}]$$

where  $n$  is the number of backcrosses.

Backcrossing of selected plants to the RP can be repeated each cycle until a line is obtained that is essentially a version of the RP that includes the introgressed allele. After six backcrosses, the expected recovery is >99% (Table 1).

Until recently, discussions of the recovery of the RP genome during backcrossing have emphasized the expected values for

%RP shown in Table 1, and have largely ignored the genetic variation for %RP that exists around the expected mean. With the development of genetic markers capable of providing good genome coverage, there has been interest in taking advantage of that variation to increase the efficiency of backcrossing.

Selection for RP marker alleles can increase greatly the effectiveness of backcross programs by allowing the breeder to 1) select backcross plants that have a higher proportion of RP genome, and 2) select backcross individuals that are better conversions near a mapped donor allele being transferred (i.e., select for less linkage drag). Expressed in practical terms, using genetic markers to assist backcrossing can 1) increase the probability of obtaining a suitable conversion, and 2) decrease the time required to achieve an acceptable recovery.

Issues to consider when planning a marker-assisted backcross program include 1) the time advantage of using markers to assist backcrossing, 2) the number of markers needed, and 3) the number of genotypes to evaluate. In this report, we use results from previous literature, computer simulation, and empirical studies to provide some guidelines.

Table 1. Expected recovery of recurrent parent (RP) genome during backcrossing, assuming no linkage to the gene being transferred.

Generation	% RP
$F_1$	50.0000
$BC_1$	75.0000
$BC_2$	87.5000
$BC_3$	93.7500
$BC_4$	96.8750
$BC_5$	98.4375
$BC_6$	99.2188
$BC_7$	99.6094

<sup>1</sup>Formerly with Purdue University, West Lafayette, Ind.

## Materials and methods

The maize genome was the model for the simulation. The simulated genome contained ten 200-cM chromosomes. Simulation of crossing over was based on a Poisson distribution with a mean of 2.0 ( $\lambda = 2$ ) (Hanson, 1959), which, on average, generated one cross over for every 100-cM length. The simulations reported here assume no interference. Codominant genetic markers were evenly distributed in the genome and sites of the donor gene were randomly assigned to genome locations.

Simulations were conducted with the following parameters:

Number of progeny: 100 or 500.

Backcross generations:  $BC_1$ ,  $BC_2$ , and  $BC_3$ .

Number of markers: 20, 40, 80, or 100.

Number selected to form the next BC generation: 1 or 5.

Selection was based on 1) presence of the donor allele and 2) high %RP. %RP was calculated as the average of the (one or five) selected individuals. Values presented are the mean of 50 simulations.

## Results

In the computer simulation study, all methods modeled greatly increased the speed of recovering the RP genome compared to the expected recovery with no marker-assisted selection (compare Tables 1 and 2). At least 80 markers were required to recover 99% of the RP genome in just three BC generations (Table 2). Use of at least 80 markers and 500 progeny allowed recovery of 98% RP in just two BC generations. Response to selection was diminished only slightly by spreading the effort over five selections. Using markers, the number of backcross generations needed to convert an Inbred is

reduced from about seven to three.

By the  $BC_3$  generation, there appears to be no practical advantage to using 500 vs. 100 individuals. If the presence of the donor trait in the backcross individuals can be ascertained before markers are genotyped, then only half the number of individuals indicated in the tables will need to be analyzed.

When a small number of markers are used, they quickly became non-informative; i.e., selection causes the marker loci to become fixed for the RP type before the rest of the genome is fully converted (Table 3; Hospital et al., 1992). This situation was most prominent in the larger populations, where a higher selection intensity placed more selection pressure upon the marker loci. Accordingly, it is of interest to consider how closely the estimation of %RP based on markers reflects the actual genome composition. The combination of estimation of %RP based on fewer markers and subsequent selection tends to bias the estimates upward (compare Tables 2 and 3).

The results from the simulation compare well with real field data. In a typical example, 50  $BC_1$  plants carrying the gene being transferred were genotyped at 83 polymorphic RFLP loci (note that this corresponds to a population size of 100 unselected plants in Tables 2 and 3). The five best  $BC_1$  recoveries had estimated %RP values of 85.9%, 82.7%, 82.0%, 81.4%, and 81.2%. After evaluating 10  $BC_2$  plants from each selected  $BC_1$ , the best  $BC_2$  recovery had an estimated %RP of 94.6%.

## Discussion

The simulations (Table 2; Hospital et al., 1992) and our experience indicate that four markers per 200-cM chromosome is adequate to greatly increase the effectiveness of selection in the  $BC_1$ . However, using only four markers per 200 cM will likely make it very difficult to map the location of the gene of interest. Adequate summarization of the data is an important

Table 2. Percent recurrent parent genome during marker-assisted backcrossing.

Generation	100 Progeny				500 Progeny			
	No. markers				No. markers			
	20	40	80	100	20	40	80	100
<i>One selected</i>								
$BC_1$	84.5	84.5	84.2	88.0	89.9	90.7	90.2	90.5
$BC_2$	95.0	95.2	95.8	97.2	96.5	97.7	98.5	98.6
$BC_3$	97.4	97.6	98.9	99.2	97.7	98.3	99.4	99.5
<i>Five selected</i>								
$BC_1$	82.9	85.1	84.9	84.7	87.7	88.1	88.9	88.9
$BC_2$	93.7	95.0	95.8	95.7	95.5	96.8	97.8	97.9
$BC_3$	97.1	98.3	98.8	98.9	97.3	98.5	99.3	99.3

Table 3. Estimates of percent recurrent parent genome, based on marker loci.

Generation	100 Progeny				500 Progeny			
	No. markers				No. markers			
	20	40	80	100	20	40	80	100
<i>One selected</i>								
$BC_2$	98.7	97.8	95.6	97.2	100.0	99.1	98.6	98.0
$BC_3$	100.0	99.8	99.3	99.5	100.0	100.0	99.9	98.2
<i>Five selected</i>								
$BC_2$	96.4	96.5	96.2	95.8	100.0	98.5	98.3	98.2
$BC_3$	99.9	99.8	99.3	99.1	100.0	100.0	99.9	99.8



part of a marker-assisted backcross program. Ideally, the markers used can supply data that can be represented as alleles of loci with known map position. Estimation of %RP, mapping the position of the locus of interest, and graphical display of the results (Young and Tanksley, 1989) are all useful in understanding and controlling the specific backcross experiment being conducted.

It appears that, with the use of genetic markers, the portion of the RP genome that is not linked to the allele being transferred can be recovered quickly and with confidence. The recovery of RP will be slower on the chromosome carrying the gene of interest. A considerable amount of linkage drag is expected to accompany selection for the DP allele in a backcross program. For a locus located in the middle of a 200-cM chromosome, the length of the DP chromosome segment accompanying selection is expected to be 126, 63, and 28 cM in the BC<sub>1</sub>, BC<sub>2</sub>, and BC<sub>3</sub> generations, respectively (Hanson, 1959; Naveira and Barbadilla, 1992). Our observations support the recommendation of Hospital et al. (1992) that preference be given to the selection for recombinants proximal to the allele of interest, but that selection for recovery of the RP elsewhere in the genome also be considered. This two-stage selection can probably be done quite effectively ad hoc by the breeder once the data is adequately summarized; however, Hospital et al.

suggest ways to incorporate the two criteria into a selection index such that each component of selection is assured appropriate weighting.

Use of genetic markers can greatly increase the effectiveness of backcrossing, and they should be used in any serious backcrossing program if resources are available to the breeder.

#### Literature Cited

- Allard, R.W. 1960. Principles of plant breeding. Wiley, New York.  
 Fehr, W.F. 1987. Principles of cultivar development: v.1. Theory and technique. Macmillan, New York.  
 Hanson, W.D. 1959. Early generation analysis of length of heterozygous chromosome segments around a locus held heterozygous with backcrossing or selfing. *Genetics* 44:843-847.  
 Hospital, F., C. Chevalet, and P. Mulsant. 1992. Using markers in gene introgression breeding programs. *Genetics* 132:1199-1210.  
 Rinke, E.H. and J.C. Sanz. 1961. Moving corn-belt germplasm northward. *Ann. Hybrid Corn Industry Conf.* 16:53-56.  
 Shaver, D.L. 1976. Conversions for earliness in maize inbreds. *Maize Genet. Coop. Nwsltr.* 50:20-23.  
 Young, N.D. and S.D. Tanksley. 1989. Restriction fragment length polymorphism maps and the concept of graphical genotypes. *Theor. Appl. Genet.* 77: 95-101.